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Short communication

Relationship Between Acrylamide Concentration and Enzymatic Activity in An Improved Single Fibrin Zymogram Gel System

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Based on the zymography analysis, *Bacillus* sp. DJ-4 (screened from Doen-Jang, a Korean traditional fermented food) secretes seven extracellular fibrinolytic enzymes (EFEs; 68, 64, 55, 45, 33, 27, and 13 kDa) in culture broth. These seven EFEs were analyzed by newly applied SDS-fibrin zymography combined with gradient polyacrylamide (SDS-FZGP). This improved gel system was used with a 5-20% acrylamide gradient in a fibrin zymogram gel for the separation of proteins with molecular masses from below 10 kDa to over 100 kDa on one gel plate. Using this system, high molecular weight bands (HMWBs) were clearly and sharply resolved. We also examined the relationship between an acrylamide concentration and the enzymatic activity of EFE using densitometric analysis.

Keywords: *Bacillus*, Extracellular protease, Fibrin zymography

Introduction

SDS-polyacrylamide gel is one of the most frequently used and powerful techniques in protein works. Gel electrophoresis of mixed proteins through a linear gradient of polyacrylamide permits an excellent resolution of a broad range (10-200 kDa) of proteins in a single gel (from 15 to 200 kDa on a 5-20% gel, or 13 to 950 kDa on a 3 to 30% gel) (Hames, 1981; Walker, 1984).

Bacillus strains are well-known for secreting various extracellular proteases, which contain wide range of proteins with molecular weights that range from 15 kDa to 100 kDa (Burg *et al*., 1990; Kobayashi *et al*., 1999; Kim and Choi, 2000; jeong and Han, 2001). In order to detect these *Bacillus* proteases, zymographic techniques are widely used (Heussen and Dowdle, 1980; Kim *et al*., 1998; Choi and Kim, 1999; Kim and Choi, 1999). These methods are based on a SDS-polyacrylamide gel that is co-polymerized with protein substrates that are degraded by the proteases, which are restored during the incubation period after an electrophoretic separation. Enzymatic activities in the zymogram gel are visualized as clear bands of fibrinolysis against a dark-blue background of undigested fibrin substrate (Kim *et al*., 1998).

In this report, we introduced an improved technique, SDS-FZGP, which combined an acrylamide gradient system with the zymogram gel for an analysis of the total extracellular fibrinolytic enzymes from *Bacillus* sp. DJ-4 was isolated from Doen-Jang, a Korean traditional fermented food. We also recommend this method as an alternative to the current technique and a routine gel electrophoretic method.

Materials and Methods

Materials Fibrinogen and thrombin from bovine for fibrin zymography were purchased from Sigma (St. Louis, USA). A gradient system (gradient mixer GM-1 and peristaltic pump P-1) was obtained from Pharmacia (USF). Other chemicals were of analytical grade.

***Bacillus* sp. DJ-4 and culture** *Bacillus* sp. DJ-4 that was isolated from Doen-Jang, a Korean traditional fermented food (Kim *et al*., 1998), was grown at 37°C in a tryptic soy broth (TSB, Difco) and transferred to 100 ml of fresh media. After a 2-day incubation, the cells were removed from the culture broth by centrifugation at 10,000 g for 10 min, and the supernatant was used for a fibrin zymographic analysis.

SDS-fibrin zymography in gradient polyacrylamide (SDS-FZGP) SDS-fibrin gel was prepared as described by Kim and Choi (Kim *et al*., 1998; Choi and Kim, 1999; Kim and Choi, 1999). The composition of the linear gradient gel (5-20%) is described in Table 1. One microgram of the supernatant from the culture broth that was diluted in a zymogram sample buffer (5 times) (consisting of 0.5 M Tris, pH 6.8, 20% SDS, 20% glycerol, and 0.03% bromophenol blue) were loaded into a fibrin gel. After running the

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Table 1. Composition of linear gradient SDS-fibrin polyacrylamide gel

Components/gel %	5%	20%
Solution A	0.82 mL	3.62 mL
Solution B	1.00 mL	1.00 mL
Bovine fibrinogen[A]	250 uL	250 uL
Bovine thrombin (1 U/mL)	50 uL	50 uL
Distiled water	2.78 mL	
Sucrose		0.75 g
SDS (10%)	50 uL	50 uL
APS (10%)	50 uL	50 uL
TEMED	5 uL	5 uL
Total	5 mL	5 mL

Solution A: 30% acrylamide, 0.8% bis-acrylamide
Solution B: 1.5 M Tris-HCl (pH 8.8)
[A]Fibrinogen (6 mg) was dissolved in solution B and then centrifuged to remove insoluble impurities

gel, it was soaked in a 2.5% Triton X-100 solution for 30 min, then incubated in a reaction buffer (30 mM Tris, pH 7.4, 200 mM NaCl, and 0.02% Brij-35) at 37°C for 12 h. The gel was stained with Coomassie blue for 1 h, then destained. The digested bands were visualized as the non-stained regions of the fibrin gel.

Densitometric analysis of digested bands For quantification, the densities of the digested bands on the zymogram gel were analyzed by video densitometry using Bio 1D ver. 97.04 (Vilber Lourmat, France) (Kleiner and Stetler-Stevenson, 1994; Choi and Kim, 2001; Choi *et al*., 2001). The protein concentration was determined according to Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as a standard.

Results and Discussion

Using a zymography analysis, it was found that *Bacillus* sp. DJ-4 secretes seven extracellular fibrinolytic enzymes (EFEs; 68, 64, 55, 45, 33, 27, and 13 kDa) in a culture broth. In gels with a high acrylamide concentration (15 and 12%), seven EFEs were detected (Fig.1 B and C). As shown in Fig. 1 (B) and (C), low molecular protein bands (LMPBs; 45, 33, 27, and 13 kDa) showed good resolution; however, it was difficult to distinguish the high molecular protein bands (HMPBs; 68, 64, and 55 kDa) in high acrylamide concentration gels (15 and 12%).

On the other hand, the gels with a relatively low acrylamide concentration (10 and 8%) demonstrated good resolution of HMPBs, but LMPBs were not detected on the gel. As shown in Fig. 1 (D), the 13 kDa band was found throughout the gel on the 10% acrylamide gel. Furthermore, in the 8% gel, two LMPBs (27 and 13 kDa) were not detected on the gel (Fig. 1, E).

In order to overcome this shortcoming, we applied a gradient gel system, which permits an excellent resolution of a broad range (10-200 kDa) of proteins in a single gel

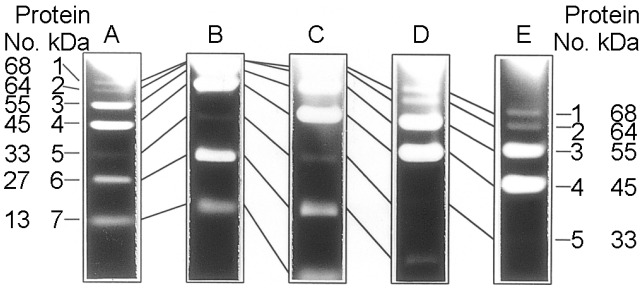

Fig. 1. Separation of the EFEs from the culture broth of *Bacillus* sp. DJ-4 on the five fibrin zymogram gel types. (A) 5-20% linear gradient gel, (B) 15% gel, (C) 12% gel, (D) 10% gel, and (E) 8% gel.

(Matsudaira and Burgess, 1978) to the fibrin zymographic technique. In the SDS-FZGP (5-20%) gel, as shown in Fig. 1 (A), up to 64 kDa bands, including two bands smaller than 30 kDa, were very well resolved in an improved gel system when compared to the corresponding polyacrylamide gels (8, 10, 12, and 15%).

We also examined the relationship between an acrylamide concentration and the enzymatic activity of EFE from *Bacillus* sp. DJ-4 using densitometric analysis (Kleiner and Stetler-Stevenson, 1994; Choi and Kim, 2001; Choi *et al*., 2001). Table 2 describes the densities of all of the EFE that was detected in the various acrylamide concentration gels. The digested HMWBs in the low acrylamide concentration gels (8 and 10%) showed higher enzymatic densities (high sensitivity in HMW area) than the other concentrations (15 and 12%); however, LMWBs were not clear. This aspect limited the visualization and distinct separation of the LMWBs (Fig. 1, D and E).

On the other hand, the gel with a relatively high acrylamide concentration (12 and 15%), as described in Table 2, revealed a low enzymatic density. But the LMWBs in these gels (12 and 15%) showed clearer bands (good distinction in LMW area) than the low acrylamide concentration gels. These results demonstrate that the activities in the zymogram gels

Table 2. Densitometric analysis of separated EFEs on the five zymogram gels

kDa/gel	Grad (5-20%)	15%	12%	10%	8%
68	3.28[a]	NS	NS	7.95	5.32
64	3.12	NS	NS	8.07	5.45
55	10.72	11.04	18.09	19.43	18.99
45	13.42	17.25	19.43	22.11	20.01
33	2.53	2.72	3.21	0.58	0.41
27	6.32	14.49	10.32	0.36	ND
13	7.58	8.32	8.57	ND	ND

a: density (cm^2)
NS: not separated band, ND: not detected band on the zymogram gel

were relayed on the acrylamide concentration, which contributed to the diffusion of proteins through the gel matrices during the incubation period in an enzyme reaction buffer after the electrophoretic separation.

An SDS-FZGP (5-20%) gel accepted these merits of high sensitivity (in the HMW area) and good distinction (in the LMW area), which were shown in the gels with low and high acrylamide concentrations, respectively (Fig. 1, A).

In this report, we applied SDS-fibrin zymography to the gradient gel for the analysis of EFE from *Bacillus* sp. DJ-4. We also described the effect of an acrylamide concentration on the enzymatic activities in zymogram gels. According to the enzymatic property, casein (for common protease), collagen (for collagenase or matrix metalloproteinase, MMP), gelatin (for gelatinase or MMP), cell wall (for cell wall hydrolase), and synthetic peptides could be used as protein substrates instead of fibrin. They could also be used in the proteomic approach.

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